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Original article

Synthesis and evaluation of artificial antigens for astragaloside IV

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The objective of this study was to produce artificial antigens for astragaloside IV that could be used to prepare antibodies against astragaloside IV screened in *Radix astragali* (*Astragalus membranaceus* (Fisch) Bunge, Fabaceae) and its preparations, using an indirect ELISA. Astragaloside IV was coupled to carrier proteins, bovine serum albumin and ovalbumin using the sodium periodate method and was then evaluated using SDS-PAGE, MALDI-TOF MS and animal immunizations. The coupling ratio of astragaloside IV to bovine serum albumin ratio was determined to be thirteen, and the indirect ELISA demonstrated that three groups of mice immunized with astragaloside IV-bovine serum albumin produced anti-astragaloside IV- bovine serum albumin-specific antibody, with a minimum serum titer of 1:9600. A method for synthesizing highly immunogenic astragaloside IV artificial antigens was successfully developed thus indicating its feasibility in the establishment of a fast immunoassay for astragaloside IV content determination in *Radix astragali* and its products.

Introduction

The saponin, astragaloside IV, a 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosyl cycloastragenol (**1**), was extracted from *Radix astragali*, the dried root of the Chinese medicinal herb *Astragalus membranaceus* (Fisch.) Bunge, Fabaceae. Pharmacological studies indicate that astragaloside IV possesses antihypertensive, positive inotropic, anti-inflammatory, antinociceptive, hepatoprotective, neuroprotective, anti-infarction, and antiviral activities, and may be effective in treating viral myocarditis. In addition, astragaloside IV has been shown to increase T, and B lymphocyte proliferation and antibody production *in vivo*, and inhibits the production of IL-1 and TNF-alpha from peritoneal macrophages *in vitro* (Duan and Sun, 2011). Astragaloside IV is increasingly being explored as an active ingredient in therapeutics; thus, there is a need to develop an effective method for its quantitation in pharmaceutical samples. Analytical methods currently used for astragaloside IV detection, include high performance liquid chromatography (HPLC), thin-layer chromatography scanning (TLCS), and fluorescence methods (Ouyang et al., 2009). However, these

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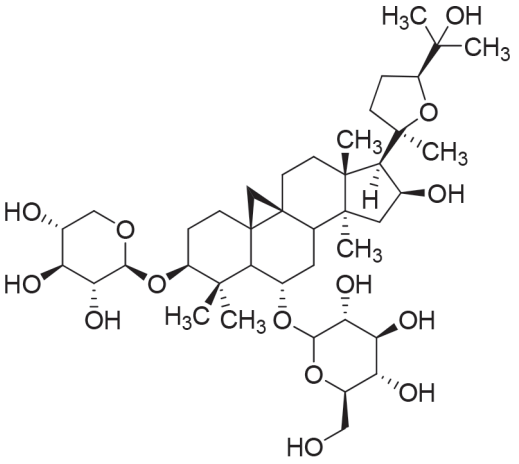
methods are complicated, mute sensitivity, require expensive instrumentation and are time-consuming. Immunoassays offer a simple, rapid, and cost-effective alternative to the traditional methods listed above, particularly when high efficiency and on-site screening tests are required. The successful preparation of artificial antigens is the first critical step in establishing any immunoassay method. Antigens (haptens) with a molecular weight (MW) less than 1000 are generally not immunogenic, and require conjugation with a carrier protein to elicit an immune response. Astragaloside IV is a relatively small molecule, with a molecular weight of 784; thus, it is not immunogenic; failing to elicit an immune response because it lacks epitopes recognized by T cells to stimulate antibody production. However, astragaloside IV, like other haptens can be chemically modified at an appropriate position to introduce a spacer with an active group at the end. The modified hapten is coupled to a macromolecular carrier to form a hapten-carrier conjugate (*e.g.* artificial antigen). The artificial antigen can make use of T cell epitopes to indirectly induce B cell proliferation and differentiation to generate specific antibodies against small molecular compounds (Jiao et al., 2004).



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In the present study, astragaloside IV was coupled to BSA to synthesize the artificial antigen, AST-BSA. The formation of AST-BSA was confirmed by SDS-PAGE and MALDI-TOF MS. An indirect ELISA was used for specificity analysis and for the determination of anti-AST-BSA specific antibody titers in serum. The results of this study laid the foundation for the preparation of monoclonal antibodies against astragaloside IV, and for the rapid detection of astragaloside IV in *Radix astragali* and its preparations using indirect ELISA.

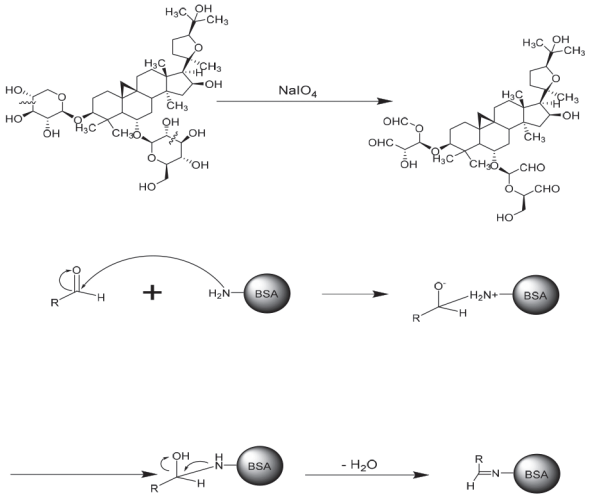
Materials and methods

Reagents and animals

Astragaloside IV standards (AST) were purchased from Chengdu Mansite Bio-Technology Co., Ltd. TMB was purchased from Solarbio Co., Ltd. Horseradish peroxidase HRP-conjugated goat anti-mouse IgG was purchased from Wuhan Boster Biological Technology Co., Ltd. Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA) were purchased from Sigma-Aldrich. The animal experimentation protocols were approved by the Animal Care and Use Committee of Yangzhou University, and all experiments were conducted according to the guidelines of Yangzhou University (Institutional Ethical Committee Number SCXK (su) 2009-0002). Female Balb/C mice (6-7 weeks old) were purchased from the Laboratory Animal Service Center of the Yangzhou University (China). The mice were kept in an animal facility in controlled conditions, temperature at 22 ± 2°C and relative humidity of 50 ± 10%. Food and water were available *ad libitum*.

Preparation of AST-carrier protein conjugates

AST-BSA and AST-OVA were prepared by the sodium periodate method (Zhao et al., 2007). Briefly, 1 ml of AST in methanol (10 mg/ml) was added by dripping to 0.06 M $NaIO_4$, stirring at room temperature. After 1 h, BSA (35.7 mg BSA in 10 ml carbonate buffer, pH 9.6) was slowly added during stirring, and the reaction incubated for 6 h at room temperature. The conjugates were lyophilized following dialysis against distilled water for three days at 4°C, and stored at -20°C. AST-OVA were prepared as for AST-BSA. Scheme 1 illustrates the synthesis of the hapten derivatives for AST.



Scheme 1 – Synthesis of hapten derivatives for astragaloside IV (R).

Analysis of AST-carrier protein conjugates by polyacrylamide gel electrophoresis

Conjugates were analyzed under denaturing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using an adaptation of the method of Joseph and David (2001). Briefly, 20 µl of conjugate (0.5 g/l) was mixed with an equal volume of 2×SDS protein loading buffer (1M Tris-HCl; pH 6.8, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and 0.02% (v/v) bromophenol blue) and let to boil for 5 min. The denatured

samples were fractionated by electrophoresis in a 5% (w/v) stacking and 15% (w/v) separating, polyacrylamide gel, with 0.1% (v/v) SDS in Tris-glycine as the buffer. Electrophoresis was conducted at 90 V and 120 V in the stacking and separating gels, respectively, and ceased once the dye reached the end of the gel. Coomassie brilliant blue R-250 solution (0.1% (w/v) in 25% (v/v) isopropyl alcohol and 10% (v/v) acetic acid) were used as gel staining agents for more than 4 h at room temperature, with gentle shaking (Kong et al., 2010). The gels were destained (50% (v/v) methanol, 10% (v/v) acetic acid) three to four times until the bands were clearly resolved. Images of the gels were taken using a UVI gel auto imaging system (Tanon Science & Technology Co., Ltd.).

Determination of the hapten/carrier protein ratio of AST-BSA by MALDI-TOF MS

Trifluoroacetic acid (TFA) 5% (30 µl) was added to 17 µl acetonitrile, then erucic acid added until the solution reached oversaturation, ultrasonicated for 15 min, and centrifuged at 112 *g* for 3 min. The solution was stored at 4°C. The AST-BSA solution (1 µl) (0.6 mg AST-BSA in 30 µl 8 mol/l urea solution diluted to the appropriate concentration, 1 mg BSA in 50 µl water) was mixed with matrix-assisted solution as the original sample. The plate was spotted with 1 µl of the original sample, point to the standard adjacent target and left to dry, and spotted 0.6 nl SA matrix, after drying, to be analyzed by MALDI TOF MS Spectrometer (AB 4800, USA). The hapten/carrier protein ratio of AST-BSA could be calculated by their respective molecular weights according to the following formula:

Conjugate proportion = $(Mr_{AST\text{-}BSA}\text{-}\ Mr_{BSA})/Mr_{AST}$ (Yang et al., 2010)

Animal immunizations

Twenty five Balb/C mice (6 weeks old) were randomly divided into five groups with five mice in each group. Three groups (Groups 1, 2, and 3) were immunized with differing amounts of AST-BSA; administered four times over a period of 45 days. The mice were injected subcutaneously with 0.2 ml (Group 1), 0.4 ml (Group 2) and 0.6 ml (Group 3) AST-BSA mixed with FCA (1:1 antigen/adjuvant) on day 1. On day 15 they were administered the same volume of AST-BSA combined with FICA (1:1 antigen/adjuvant). On days 30 and 45, the mice were administered the same volume of AST-BSA alone (Boesen et al., 2005). The fourth group (negative control) was immunized with BSA as per the experimental groups, and the fifth group (blank control) was left untreated. Three days after the last injection the mice were bled from the eye and the serum antibody titers determined using an indirect ELISA (Shin et al., 2006).

Indirect ELISA for the detection of anti-AST-BSA antibodies

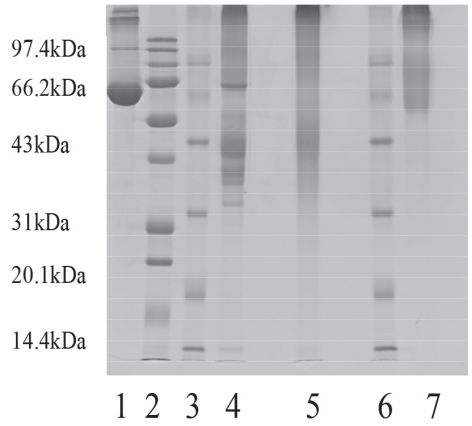
The indirect ELISA was conducted in Corning 96 well microtiter plates (Corning, NY) coated with 1.25 µg/mg AST-OVA in 50 mM carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed three times with 0.05% Tween 20 in PBS (PBST) and non-specific protein binding sites were blocked with 1% (w/v) skim milk powder in PBS at 37°C for 1 h. Serum obtained from the three groups of mice immunized with AST-BSA, the negative control group (saline) and blank control (normal mouse serum) were diluted 1:1000 to 1:32000 in PBS, and 100 µl added to the wells of the plate, tests were performed in triplicate. The plates were incubated for 60 min at 37°C, washed with PBST, and a 1:5000 dilution of HRP-conjugated goat anti-mouse IgG in PBS added to each well. The plates were incubated for 60 min. at 37°C, washed, and TMB substrate solution (0.2 mg/ml TMB and 50 µl 30% H_2O_2, in 10 ml 0.1M citrate-0.2M phosphate buffer, pH 5.2) added. The plates were incubated for 30 min at room temperature and the reaction stopped with 50 µl 2M H_2SO_4. The optical density was measured at a wavelength of 450 nm using the BioTek ELx800 ELISA reader (Wei et al., 2012). Each sample was replicated three and the average value was used as the final value.

Results and discussion

Analysis of AST-carrier protein conjugates by SDS-PAGE.

Proper hapten design was essential for antibody production and ELISA development. AST is a small molecular compound (MW of 784 kDa), and as such is not immunogenic. In order for AST (and other haptens) to elicit an immune response it must be conjugated to a carrier protein. The haptens must contain functional groups, or be easily derivatized, to incorporate reactive groups (such as amino, carboxyl, diazo group) for cross-linking reactions with the protein (Song et al., 2010). The carboxyl and hydroxyl groups on hydroxylated haptens must be activated for cross-linking with carrier proteins. AST is polyhydroxy and consequently the first reaction with sodium periodate is the oxidation of the polyhydroxy group to react to aldehyde and carboxyl groups, which conjugate with the amino group of BSA through an amide bond, to generate the AST-BSA protein complex. Normally, UV spectroscopy is used to identify if the hapten has been conjugated to the carrier protein, however, AST has no absorption in the ultraviolet region, thus, and in the current study we used protein electrophoresis to evaluate the successful coupling of AST to the carrier protein BSA preliminarily.

The results of the SDS-PAGE analysis of AST-BSA and AST-OVA conjugates are presented in Fig. 1. As shown, the



Lane 1 BSA sample 2 MarkerI 3 MarkerII 4 OVA sample 5 AST-OVA sample 6 MarkerII 7 AST-BSA sample.

Figure 1 – The results of non-denaturing polyacrylamide gel electrophoresis.

electrophoretic migration pattern of the AST-carrier proteins, AST-BSA and AST-OVA, differ from their respective carrier proteins, OVA and BSA. As expected, the electrophoretic mobility of BSA was greater than that of AST-BSA, and similarly, greater for OVA than that for AST-OVA. These results are consistent with the greater molecular weights of AST-BSA and AST-OVA compared with BSA and OVA, respectively. These results demonstrate the artificial antigens, AST-BSA and AST-OVA, were produced successfully.

Determination of the hapten/carrier protein ratio of AST-BSA by MALDI TOF MS

As the titer of antibody serum is often affected by the ratio of hapten/carrier, it was necessary to detect the ratio of hapten/carrier before immunizing the mice. Hapten ratios for the conjugates could not be measured by UV spectroscopy by the reason above-mentioned that AST has no absorption in the ultraviolet region, so the prepared AST-BSA conjugate product was examined by MALDI TOF MS to detect the hapten/carrier protein ratio. The identification of BSA and AST-BSA conjugates by MALDI TOF MS are presented in Fig. 2 and Fig. 3, respectively. The molecular weight of BSA was determined as 66,446.4766 kDa (Fig. 2) and the molecular weight of the AST-BSA conjugate as 76,970.9609 (Fig. 3). The hapten/carrier protein ratio was determined as 13.4 by the formula: Conjugate proportion = ($Mr_{AST-BSA-}$ Mr_{BSA})/Mr_{AST}) [(76970.9609-66446.4766)/66446.4766≈13.4:1]. Accordingly, the hapten/carrier protein ratio is approximately 13:1. According to the literature, hapten/carrier ratios of 3:1-45:1 indicate artificial antigens with strong immunogenicity (Wang et al., 2011). So the AST-BSA binding ratio of 13.4 established in the current study suggests that coupling conditions used for the hapten and the carrier successfully generate a strong immunogen.

Determination of serum anti-AST-BSA-specific antibody titers

The optimum concentrations of antigen were determined and the serum anti-AST-BSA-specific antibody titers of mice immunized with AST-BSA conjugates were measured using an indirect ELISA to confirm the AST-BSA conjugate has a strong immunogenicity. As the mice were immunized with AST-BSA when performing an indirect ELISA, AST-OVA were selected to coat the plate in order to avoid the effect of the antibody in the serum capable to conjugate with BSA. BSA is very immunogenic and it will be able to present a large number of cross-reactions so the serum from mice immunized with BSA was selected as a negative control in order to avoid a false positive reaction. The optical density (OD) for the control serum was significantly lower than the OD for test serum ($p < 0.05$). Final values are presented as the average of three replicates ± the standard deviation. The titers of anti-sera for groups 3 (immunized with 0.6 ml AST-BSA per time), 2 (immunized with 0.4 ml AST-BSA per time), and 1 (immunized with 0.2 ml AST-BSA per time) were 25,600, 12,800 and 9,600, respectively (Fig. 4). These results demonstrate that the conjugates had good immunogenicity. It can then be concluded that immunization of Balb/C mice with artificial antigen produced high anti-AST-BSA-specific serum titers, as measured by the indirect ELISA. This experiment lays the foundation to assay AST using an indirect ELISA approach.

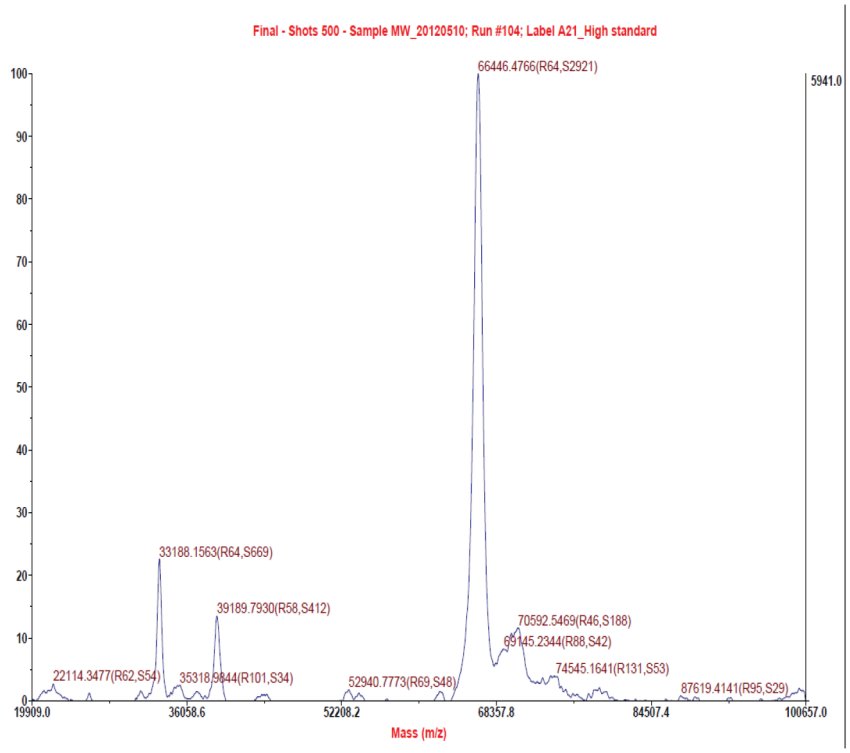


Figure 2 – Identification of BSA by MALDI-TOF MS.

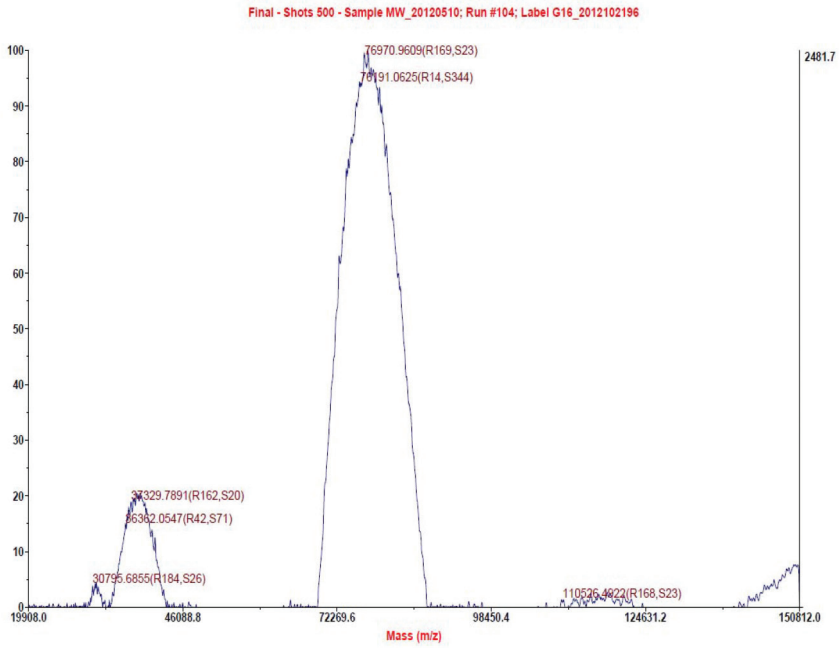


Figure 3 – Identification of astragaloside IV-BSA by MALDI-TOF MS.

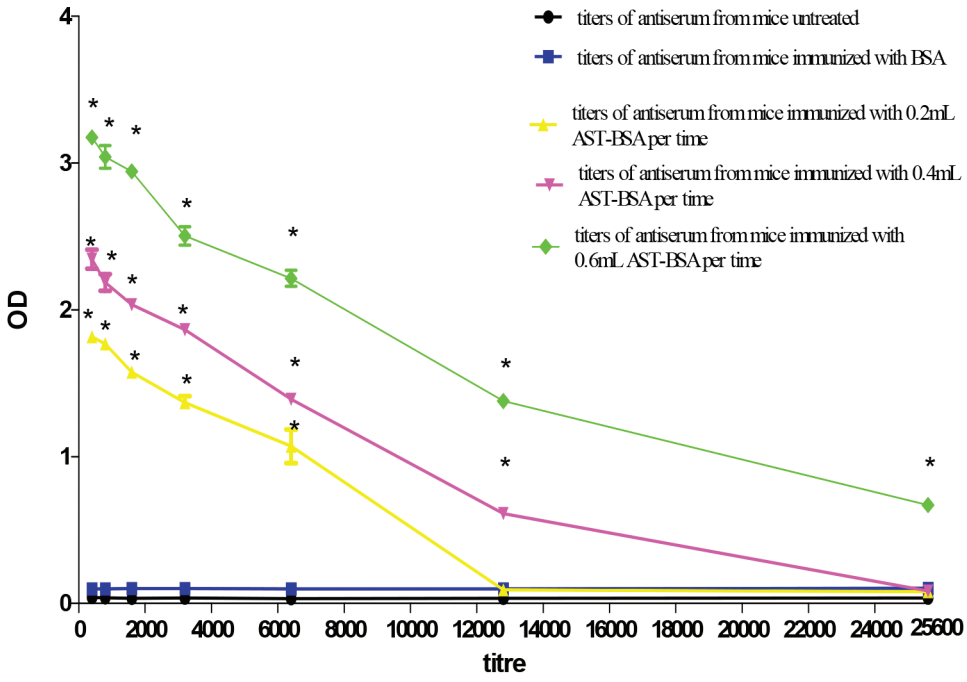


Figure 4 – Serum anti-AST-BSA antibody titers from immunized mice.

Authors' contributions

SY performed the evaluation of artificial antigens, analysis of the data and drafted the paper. JX produced artificial antigens, running the laboratory work. TM contributed to critical reading of the manuscript. ZO designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved for submission.

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Conflicts of interest

The authors declare no conflicts of interest.

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